Impaired NO-dependent inhibition of store- and receptor-operated calcium entry in pulmonary vascular smooth muscle after chronic hypoxia

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ENDOTHELIUM-DERIVED NITRIC OXIDE (EDNO) may play an important protective role in diminishing the severity of chronic hypoxia (CH)-induced pulmonary hypertension (9, 35). CH is associated with enhanced EDNO-dependent vasodilation, a function of elevated endothelial nitric oxide synthase expression (9, 20, 28, 29, 32, 37) and increased pulmonary vascular smooth muscle (VSM) sensitivity to nitric oxide (NO) (16). Moreover, this enhanced VSM reactivity to NO following CH correlates with increased expression and activity of the downstream effector, protein kinase G (PKG) (15). Interestingly, the augmented NO-dependent vasodilation after CH is coupled to a smaller decrease in VSM intracellular free calcium ([Ca^{2+}]_i) compared with vessels from control animals (16), suggesting that CH promotes a change in NO signaling resulting in less dependence on Ca^{2+}-handling pathways such as Ca^{2+} sequestration, influx, or efflux and greater dependence on mechanisms that regulate Ca^{2+} sensitivity. Consistent with this possibility, recent studies from our laboratory indicate that spermine NONOate reverses Ca^{2+} sensitization generated by the small G protein, RhoA, and Rho kinase (ROK) in permeabilized small pulmonary arteries from CH but not control animals (17). However, the mechanism by which CH impairs NO-dependent decreases in VSM [Ca^{2+}]_i remains to be investigated and is the focus of the current study.

NO-mediated stimulation of PKG elicits relaxation through several mechanisms that result in either a decrease in [Ca^{2+}]_i or a decrease in the sensitivity of the contractile apparatus to Ca^{2+} (2). Ca^{2+} entry into VSM cells is mediated by voltage-operated Ca^{2+} channels (30) as well as several types of Ca^{2+}-permeable channels that are not voltage gated. These include channels mediating store-operated Ca^{2+} entry (SOCE), activated by depletion of Ca^{2+} from the sarcoplasmic reticulum (SR), and receptor-operated Ca^{2+} entry (ROCE), commonly activated by agonist stimulation of G protein-coupled receptors (23). We hypothesized that CH impairs NO-mediated decreases in pulmonary VSM [Ca^{2+}]_i by interfering with PKG-dependent inhibition of either SOCE or ROCE. To test this hypothesis, we measured changes in [Ca^{2+}]_i, and inner diameter (ID) elicited by SOCE and ROCE in isolated, endothelium-denuded, pressurized small pulmonary arteries from CH and control rats. In addition, we examined effects of the NO donor spermine NONOate on SOCE and ROCE in vessels from control, but not CH animals. We conclude that NO-mediated inhibition of SOCE and ROCE is impaired after CH-induced pulmonary hypertension.

METHODS

All protocols and surgical procedures employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine (Albuquerque, NM).

Experimental groups. Male Sprague-Dawley rats (200–300 g body wt, Harlan Industries) were divided into two groups for each experiment. Animals designated for exposure to CH were housed in a hypobaric chamber with barometric pressure maintained at ~380 mmHg for 4 wk. The chamber was opened three times per week to provide animals with fresh food, water, and clean bedding. On the day of experimentation, rats were removed from the hypobaric chamber and immediately placed in a Plexiglas chamber continuously flushed with a 12% O_2-88% N_2 gas mixture to reproduce inspired P_O_2 (~70 mmHg) within the hypobaric chamber. Control animals were housed at ambient barometric pressure (~630 mmHg). All animals were maintained on a 12:12-h light-dark cycle.

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Endothelial disruption and cannulation of small pulmonary arteries for dimensionality analyses. Rats were anesthetized with pentobarbital sodium (200 mg/kg ip), and the heart and lungs were exposed by midline thoracotomy. The left lung was removed and immediately placed in ice-cold physiological saline solution (PSS) containing (in mM) 129.8 NaCl, 5.4 KCl, 0.83 MgSO4, 19 NaHCO3, 1.8 CaCl2, and 5.5 glucose. The lung was pinned out in icd PSS in a Silastic-coated dissection dish. A fourth or fifth order intrapulmonary artery (~100- to 300-μm ID) of ~1-mm length and without visible side branches was dissected free and transferred to a vessel chamber (Living Systems, CH-1) containing icd PSS. The proximal end of the artery was cannulated with a tapered glass pipette, secured in place with a single strand of silk ligature, and gently flushed to remove any blood from the lumen. The vessel was monitored during this time to ensure blood exited the distal end and not through small openings in the length of the vessel that may be indicative of side branches. Before the distal end of the artery was cannulated, the vessel lumen was rubbed with a strand of moose mane to disrupt the endothelium. The vessel was then stretched longitudinally to approximate its in situ length and pressurized with either a column or a servo-controlled peristaltic pump. In addition to the above criteria, these arteries were required to hold a steady pressure upon switching off the servo-control function to confirm the absence of a leak. Any vessels with apparent leaks were discarded. The vessel chamber was transferred to the stage of a Nikon Eclipse TS100 microscope where vessels from both groups were superfused with PSS equilibrated with a 20% solution of Pluronic acid in DMSO, and this mixture was continuously present in the superfuse. 

**Measurement of VSM [Ca2+]i.** Pressurized arteries were loaded abuminally with the cell-permeant, ratiometric, Ca2+-sensitive fluorescent indicator fura-2 AM (Molecular Probes). Immediately before being loaded, fura-2 AM (1 mM in anhydrous DMSO) was mixed 2:1 with a 20% solution of Pluronic acid in DMSO, and this mixture was diluted in PSS to yield a final concentration of 2 μM fura-2 AM and 0.05% Pluronic acid. Arteries were incubated in this solution for 45 min at room temperature in the dark. The diluted fura-2 AM solution was equilibrated with the 10% O2 gas mixture during this loading period. Vessels were rinsed for 20 min with aerated PSS (37°C) after any residual Ca2+ response due to ATP-mediated citrulinemia to prevent Ca2+ entry through L-type voltage-gated Ca2+ channels. The efficacy of diltiazem was verified in control arteries by blockade of the [Ca2+]i response to a depolarizing concentration of KCl (50 mM). In addition, arteries were incubated with the SR Ca2+-ATPase (SERCA) inhibitors cyclopiazonic acid (CPA; 10 μM) or thapsigargin (TG; 1 μM) to deplete intracellular Ca2+ stores and prevent Ca2+ reuptake. The changes in [Ca2+]i (SOCE) and ID were then determined upon restoration of extracellular Ca2+ (1.8 mM) in the continued presence of diltiazem and CPA or TG. Separate sets of arteries from each group were pretreated with the relatively nonselective inhibitor of Ca2+ entry, NiCl2 (10 mM) (40). Parallel experiments were performed in the presence of SKF-96365 (50 μM), which has been reported to selectively inhibit ROCE vs. SOCE (8, 12, 25).

**Effect of CH on SOCE.** Effects of the NO donor, spermine NONOate (1 μM), on CPA-induced SOCE and vasoconstriction were assessed in vessels from each group as above. Spermine NONOate was added and was independently present in both Ca2+-free and normal PSS. [Ca2+]i responses to spermine NONOate (1 μM) were further determined in the presence of the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 50 μM) or the PKG inhibitor Rp-8-Br-PET-cGMP (30 μM). Additional experiments examined responses to the PKG agonist 8-pCPT-cGMP (10 μM) in separate sets of control and CH vessels.

**Effects of CH and NO on Mn2+ quenching of fura-2 fluorescence.** Store-operated cation entry was quantified by the rate of quenching of fura-2 fluorescence with Mn2+, which enters the VSM as a Ca2+- surrogate and reduces fura-2 fluorescence on binding to the dye. Pulmonary arteries were loaded with fura-2, as previously described, and washed with aerated PSS for 20 min. Fura-2 was excited at 360 nm, and emission light was recorded at 510 nm. At the excitation wavelength of 360 nm, fura-2 fluorescence intensity is not influenced independently of SOCE and to eliminate other UTP-mediated Ca2+ entry mechanisms, vessels were pretreated with diltiazem (50 μM) and CPA (10 μM) for 15 min. MnCl2 (500 μM) was then added to the superfuse for 10 min. In experiments examining the effect of NO on store-operated cation entry, spermine NONOate (1 μM) was continuously present in the superfuse.

**Effects of CH and NO on ROCE.** ROCE was induced with the G protein-coupled P2Y receptor agonist UTP (14). To assess ROCE independently of SOCE and to eliminate other UTP-mediated Ca2+ entry mechanisms, vessels were pretreated with diltiazem (50 μM) and CPA (10 μM), and a stable SOCE response was obtained (described above) before the addition of UTP (100 μM). Parallel protocols were performed in arteries from each group pretreated with NiCl2 (10 mM) and SKF-96365 (50 μM). Similar experiments were conducted in the presence of spermine NONOate (1 μM) to examine potential inhibitory effects of NO on UTP-induced Ca2+ entry through receptor-operated channels.

**Calculations and statistics.** Vasoconstrictor responses were calculated as a percent of baseline ID for SOCE experiments and as a percent of ID after SOCE for ROCE experiments. All data are expressed as means ± SE, and values of n refer to the number of animals in each group. A t-test or two-way ANOVA was used to make comparisons when appropriate. If differences were detected by ANOVA, individual groups were compared with the Student-Newman-Keuls test. P ≤ 0.05 was accepted as significant for all comparisons.

**RESULTS**

**Efficacy of CPA, TG, and diltiazem.** To assess influences of CH on SOCE, the SERCA inhibitors CPA and TG were used to deplete intracellular Ca2+ stores and thereby activate store-operated cation channels, whereas diltiazem was employed to

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eliminate any contribution of Ca\(^{2+}\) influx through L-type voltage-dependent Ca\(^{2+}\) channels. Representative traces illustrating the efficacy of these compounds in isolated control arteries are depicted in Fig. 1, A and B. UTP elicited a transient increase in VSM \([\text{Ca}^{2+}]_{i}\) \((\Delta \text{ in } F_{340}/F_{380} = 0.08 \pm 0.01; n = 3)\) associated with a transient constriction \((40\% \pm 3\%; n = 3)\) in Ca\(^{2+}\)-free PSS, signifying Ca\(^{2+}\) release from intracellular stores (Fig. 1A, left). Repeating this protocol in the presence of either CPA (Fig. 1A, middle) or TG (Fig. 1A, right) largely inhibited UTP-mediated changes in \([\text{Ca}^{2+}]_{i}\) \((\Delta \text{ in } F_{340}/F_{380} = -0.01 \pm 0.01, n = 4 \text{ for CPA}; \text{ and } 0.00 \pm 0.01, n = 3 \text{ for TG})\) and ID \((7\% \pm 2\%, n = 4 \text{ for CPA}; \text{ and } 3\% \pm 1\%, n = 3 \text{ for TG})\), thus demonstrating effective depletion of intracellular Ca\(^{2+}\) stores by these inhibitors.

Figure 1B illustrates that KCl \((50 \text{ mM})\)-induced increases in \([\text{Ca}^{2+}]_{i}\) \((\Delta \text{ in } F_{340}/F_{380} = 0.38 \pm 0.03, n = 3)\) were diminished by 50 \(\mu\text{M}\) diltiazem \((\Delta \text{ in } F_{340}/F_{380} = 0.02 \pm 0.01, n = 3)\). Diltiazem similarly prevented KCl-induced vasoconstriction \((76\% \pm 10\%, n = 3 \text{ without diltiazem}; \text{ and } 3\% \pm 3\%, n = 3 \text{ with diltiazem}), \text{ indicating blockade of voltage-gated Ca}^{2+} \text{ channels.}

CH inhibits SOCE. Baseline ID and \([\text{Ca}^{2+}]_{i}\) were similar between groups (Table 1). Figure 2A depicts traces of VSM \([\text{Ca}^{2+}]_{i}\) and ID from individual control arteries, indicating SOCE (left) and blockade of SOCE and associated vasoconstriction by NiCl\(_2\) (middle) but not the putative ROCE inhibitor SKF-96365 (right).

SOCE was significantly attenuated in vessels isolated from CH rats compared with controls after SERCA inhibition with
Similar results were obtained for TG-induced SOCE (Table 2). Although the change in $[Ca^{2+}]_i$ was diminished after CH, the percent changes in ID were not different between groups (Fig. 2C, Table 2). SOCE and associated vasoconstriction were blocked with NiCl$_2$, but not SKF-96365, in each group (Fig. 2, Table 2). Furthermore, CH-induced SOCE remained attenuated in the presence of SKF-96365 (Fig. 2B).

CH impairs NO-dependent inhibition of SOCE. Treatment with either the NO donor spermine NONOate or the cGMP analog 8-pCPT-cGMP attenuated SOCE in control, but not CH, pulmonary arteries (Fig. 3A). Although there is a tendency for both spermine NONOate and 8-pCPT-cGMP to inhibit SOCE-induced vasoconstriction in control vessels, these differences did not achieve statistical significance (Fig. 3B). Furthermore, both the soluble guanylyl cyclase inhibitor ODQ and the PKG inhibitor Rp-8-Br-PET-cGMPS prevented spermine NONOate-induced attenuation of SOCE in vessels from control animals (Fig. 3A), suggesting NO inhibits SOCE through a cGMP/PKG-dependent mechanism.

### Table 1. Baseline inner diameter and vascular smooth muscle $[Ca^{2+}]_i$ of pulmonary arteries from control and chronically hypoxic rats

<table>
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<tr>
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<th>Control</th>
<th>Chronic Hypoxia</th>
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<tr>
<td>Baseline inner diameter, μm</td>
<td>213 ± 8 (n=57)</td>
<td>213 ± 7 (n=52)</td>
</tr>
<tr>
<td>Baseline $[Ca^{2+}]<em>i$ (F$</em>{340}/$F$_{380}$)</td>
<td>0.91 ± 0.03 (n=57)</td>
<td>0.84 ± 0.03 (n=52)</td>
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</table>

Values are means ± SE. There are no significant differences. $n$, Number of animals; F$_{340}/$F$_{380}$, fura-2 340/380-nm emission ratio.

### Table 2. Chronic hypoxia inhibits thapsigargin-induced store-operated Ca$^{2+}$ entry

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Chronic Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[Ca^{2+}]<em>i$ (F$</em>{340}/$F$_{380}$)</td>
<td>0.416 ± 0.036</td>
<td>0.242 ± 0.045*</td>
</tr>
<tr>
<td>NiCl$_2$</td>
<td>0.046 ± 0.012#</td>
<td>0.042 ± 0.014#</td>
</tr>
<tr>
<td>SKF-96365</td>
<td>0.370 ± 0.052</td>
<td>0.220 ± 0.048*</td>
</tr>
<tr>
<td>% Vasoconstriction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>39.2 ± 11.0</td>
<td>29.4 ± 12.1</td>
</tr>
<tr>
<td>NiCl$_2$</td>
<td>0.4 ± 0.5#</td>
<td>−0.2 ± 0.9#</td>
</tr>
<tr>
<td>SKF-96365</td>
<td>28.6 ± 12.4</td>
<td>16.4 ± 8.4</td>
</tr>
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</table>

Values are means ± SE. $n = 5/Treatment/group. *P ≤ 0.05 vs. control. #P ≤ 0.05 vs. vehicle. Δ Vascular smooth muscle $[Ca^{2+}]_i$ (F$_{340}/$F$_{380}$) and associated vasoconstriction (% baseline inner diameter) in endothelium-denuded small pulmonary arteries from control and chronically hypoxic rats after restoration of extracellular Ca$^{2+}$. All experiments were performed in the presence of thapsigargin (1 μM) and diltiazem (50 μM) plus either vehicle (PSS), NiCl$_2$ (10 mM), or SKF-96365 (50 μM).
cGMPS was without effect on SOCE in CH vessels (Fig. 3A) but augmented vasoconstrictor responses to SOCE in this group (Fig. 3B).

**CH attenuates both store-operated Mn2⁺ influx and NO-mediated inhibition of Mn2⁺ entry.** Similar to effects of CH on SOCE, Mn2⁺ quenching of fluorescence was markedly decreased in arteries from CH rats vs. controls (Fig. 4). Spermine NONOate inhibited Mn2⁺ influx in control arteries but was without effect in vessels from CH rats, thus providing additional support for an effect of CH to impair NO-dependent inhibition of SOCE.

**CH inhibits ROCE.** Figure 5A depicts traces for VSM [Ca²⁺]i and ID in individual control vessels, demonstrating UTP-induced ROCE after induction of stable SOCE. Whereas NiCl₂ blocked SOCE, UTP-dependent increases in [Ca²⁺]i persisted in the presence of this inhibitor, suggesting specificity of NiCl₂ for SOCE vs. ROCE. In contrast, the putative ROCE inhibitor SKF-96365 prevented UTP-dependent [Ca²⁺]i responses but was without effect on SOCE.

Similar to effects of CH on SOCE, UTP-induced ROCE (Fig. 5B) was attenuated in CH vessels compared with controls. However, despite CH-induced inhibition of ROCE, vasoconstrictor responses to UTP were not different between groups (Fig. 5C). NiCl₂ inhibited UTP-dependent changes in [Ca²⁺]i and vasoconstriction in control vessels but did not alter responses in CH arteries. In contrast, SKF-96365 abolished UTP-induced changes in [Ca²⁺]i and largely diminished vasoconstrictor responses in both groups. Spermine NONOate provided a modest but significant inhibition of ROCE in control arteries, although vasoconstrictor responses to UTP were unaltered by this NO donor. Whereas spermine NONOate was without effect on UTP-induced changes in [Ca²⁺]i, in CH arteries, there was a significant decrease in UTP-mediated vasoconstriction, which may be a consequence of increased spermine NONOate-induced Ca²⁺ desensitization after CH (17).

**DISCUSSION**

Previous studies from our laboratory have demonstrated that long-term hypoxia impairs NO-mediated decreases in VSM [Ca²⁺]i in isolated small pulmonary arteries (16). The present study examined whether CH mediates this response by interfering with NO-dependent inhibition of SOCE and ROCE in pulmonary VSM. The major findings from this study are: 1) CH attenuates SOCE and ROCE; and 2) NO diminishes SOCE and ROCE in control arteries; however, these inhibitory effects of NO are absent after CH. These results, summarized in Fig. 6, suggest that hypoxic acclimation leads to diminished NO-dependent decreases in pulmonary VSM [Ca²⁺]i, at least in part...
by interfering with NO-mediated inhibition of SOCE and ROCE.

Relaxation to NO/PKG can be elicited either by lowering [Ca^{2+}]_{i} or by decreasing the sensitivity of the contractile apparatus to Ca^{2+} in pulmonary arteries from control rats, whereas PKG-mediated decreases in VSM [Ca^{2+}]_{i} are diminished after long-term hypoxia (16). This latter effect of CH to interfere with NO regulation of Ca^{2+} handling mechanisms in pulmonary VSM is compensated by an increase in PKG-dependent Ca^{2+} desensitization, which appears to be largely attributed to inhibition of RhoA/ROK (17). These recent findings have led us to examine CH-induced alterations to mechanisms of VSM Ca^{2+} handling in the pulmonary circulation.

Activation of many G protein-coupled receptors mediates Ca^{2+} influx in VSM through both receptor-operated and store-operated cation channels (27). Whereas receptor-operated channels can be stimulated via phospholipase C-derived diacylglycerol, store-operated channels are activated secondary to depletion of Ca^{2+} from the SR, a process termed capacitative Ca^{2+} entry or SOCE (23). SOCE has been demonstrated in isolated pulmonary arterial smooth muscle cells (7, 11, 26, 36, 39, 40) and in VSM from both conduit (22) and distal pulmonary arteries (34). Snitkov et al. (34) have shown that SOCE occurs in small arteries from different beds (mesenteric, renal, femoral, and pulmonary), although the coupling of SOCE to vasoconstriction was observed only in intrapulmonary arteries, demonstrating the potential significance of this Ca^{2+} entry mechanism in regulation of pulmonary vascular reactivity. Our present finding that capacitative Ca^{2+} entry mediates vasoconstriction in isolated small pulmonary arteries is in agreement with these earlier observations. We have further identified a novel effect of CH to attenuate both SOCE and ROCE. Interestingly, the vasoconstriction associated with each Ca^{2+} entry pathway was similar between groups, consistent with recent evidence from our laboratory for enhanced pulmonary VSM Ca^{2+} sensitivity after CH (17). However, neither the mechanisms by which CH inhibits SOCE and ROCE nor the physiological significance of these responses is understood.

The best candidates for store-operated and receptor-operated channels are several members of the canonical transient receptor potential (TRPC) family of cation channels (41). Although first characterized as light-activated cation channels in photoreceptor cells of Drosophila (13), mammalian TRPC ion channels have recently gained recognition for their involvement in...
SOCE and ROCE. Of the seven known TRPC channels, denoted TRPC1–7, expression of TRPC1–6 has been identified by RT-PCR (21, 22, 26, 38, 39) in rat pulmonary arterial smooth muscle cells. The role of TRPC channels in SOCE has been evaluated in cell culture by either overexpression of TRPC protein or interference with expression by genetic manipulations [antisense oligonucleotides, small interfering (si) RNA, or gene knockout]. In human pulmonary artery smooth muscle cells, SOCE and cationic current were significantly reduced when treated with TRPC1 antisense (36). Moreover, Lin et al. (21) demonstrated that siRNA knockdown of TRPC1 attenuates thapsigargin-induced Ca$^{2+}$ entry, whereas knockdown of TRPC6 inhibits influx of Ca$^{2+}$ mediated by the diacylglycerol analog 1-oleoyl-2-acetyl-sn-glycerol. Therefore, TRPC1 and TRPC6 appear to contribute to SOCE and ROCE, respectively, in pulmonary VSM.

It is possible that diminished SOCE and ROCE after CH observed in the current study is a result of decreased expression or activity of one or more TRPC channel proteins. At odds with this hypothesis, however, Lin and colleagues (21) have observed enhanced SOCE and ROCE in transiently cultured pulmonary myocytes from CH rats compared with controls, which correlates with an upregulation of both TRPC1 and TRPC6 expression. The reason for these apparent discrepancies between the current study and earlier work (21) is unclear but may be a consequence of the different preparations used. One possible explanation is that the enzymatic tissue digestion and cell culture procedures used previously (21) resulted in differential alterations in cellular phenotype between groups. A further possibility is that segmental heterogeneity exists in pulmonary myocytes from CH rats compared with controls, which correlates with an upregulation of both TRPC1 and TRPC6 expression. Nevertheless, an effect of CH to elevate basal cytosolic free Ca$^{2+}$ would not be predicted based on our present findings of diminished SOCE and ROCE after CH.

Despite the lack of NO-mediated inhibition of SOCE in CH vessels, there was a significant PKG-dependent inhibitory effect of NO on SOCE in arteries from control animals. NO can inhibit SOCE indirectly via inactivation of phospholamban, subsequent stimulation of SERCA, and increased filling of sarcoplasmic reticulum (SR) Ca$^{2+}$ stores with the SR Ca$^{2+}$-ATPase (SERCA) inhibitors CPA or TG. Ca$^{2+}$ entry via receptor-operated channels (ROC) was elicited by the P2Y receptor agonist UTP after SOCE. A: control arteries. NO inhibited SOCE in pulmonary VSM via a cGMP/PKG pathway (solid line). NO provided a modest inhibition of ROCE, although the signaling pathway mediating this effect has not been defined (dashed line). NiCl$_2$ prevented SOCE (solid line) and inhibited ROCE (dotted line), whereas SKF-96365 blocked only ROCE. B: CH arteries. CH attenuated both SOCE and ROCE. NO was without effect on either Ca$^{2+}$ influx pathway. NiCl$_2$ and SKF-96365 selectively inhibited SOCE and ROCE, respectively. These data support a shift in NO/PKG signaling from [Ca$^{2+}$],-lowering mechanisms to those involving Ca$^{2+}$ desensitization via inhibition of the RhoA/Rho kinase (ROK) pathway in CH arteries as previously reported (17).
SR Ca$^{2+}$ stores (3–5, 18, 24). In the current study, however, it is likely that NO/PKG attenuated SOCE independently of the SR Ca$^{2+}$ load, given that SERCA was continuously inhibited with CPA. A similar direct effect of NO to inhibit capacitative Ca$^{2+}$ entry has been suggested in bovine endothelial cells (6). One possible explanation for these findings is that NO/PKG mediates a direct inhibitory effect on the channels involved in SOCE, presumably members of the TRPC family of cation channels (41). Consistent with this possibility is evidence that PKG can directly phosphorylate TRPC3 channels in HEK-293 cells leading to diminished SOCE (19). Although our findings support an effect of PKG to inhibit SOCE independently of functional coupling to the SR Ca$^{2+}$ load in control arteries, they do not preclude a potential role for increased SERCA activity in mediating NO-induced pulmonary vasodilation in this preparation. However, neither the mechanism by which PKG stimulation leads to inhibition of capacitative Ca$^{2+}$ entry in pulmonary VSM nor the mechanism by which long-term hypoxia attenuates this response is understood; both represent viable areas of future investigation.

Similar to effects of CH on NO-dependent regulation of SOCE, we observed a unique role for CH to prevent NO-mediated inhibition of SOCE. Together, these results suggest that the diminished effect of NO to reduce cytosolic [Ca$^{2+}$] previously observed in CH pulmonary VSM (16, 17) is mediated by a lack of NO-dependent attenuation of both ROCE and SOCE. Although these results may be explained by a derangement in NO signaling following CH, it is alternatively possible that the inability of NO to inhibit Ca$^{2+}$ entry in CH arteries is a function of already diminished SOCE and ROCE. Further study will be required to determine whether CH is having additional effects to alter other mechanisms of NO signaling involving regulation of Ca$^{2+}$ sequestration, influx, and efflux.

It is noteworthy that SKF-96365 inhibited UTP-induced ROCE, whereas it failed to affect Ca$^{2+}$ entry in control arteries (8). In contrast, other reports have demonstrated inhibition of SOCE by SKF-96365 in isolated pulmonary arteries. Therefore, NiCl$_2$ may have additional effects to alter other mechanisms of NO signaling involving regulation of Ca$^{2+}$ sequestration, influx, and efflux.

It is noteworthy that SKF-96365 inhibited UTP-induced [Ca$^{2+}$]$_i$ previously observed in CH pulmonary VSM (16, 17) is mediated by a lack of NO-dependent attenuation of both ROCE and SOCE. Although these results may be explained by a derangement in NO signaling following CH, it is alternatively possible that the inability of NO to inhibit Ca$^{2+}$ entry in CH arteries is a function of already diminished SOCE and ROCE. Further study will be required to determine whether CH is having additional effects to alter other mechanisms of NO signaling involving regulation of Ca$^{2+}$ sequestration, influx, and efflux. It is noteworthy that SKF-96365 inhibited UTP-induced [Ca$^{2+}$]$_i$ previously observed in CH pulmonary VSM (16, 17) is mediated by a lack of NO-dependent attenuation of both ROCE and SOCE. Although these results may be explained by a derangement in NO signaling following CH, it is alternatively possible that the inability of NO to inhibit Ca$^{2+}$ entry in CH arteries is a function of already diminished SOCE and ROCE. Further study will be required to determine whether CH is having additional effects to alter other mechanisms of NO signaling involving regulation of Ca$^{2+}$ sequestration, influx, and efflux.

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